THE ACTION OF PHOSPHOLIPASE A_2 PURIFIED FROM *CROTALUS ATROX* VENOM ON SPECIFICALLY LABELLED 2-ACYL-1-ALK-1′-ENYL- AND 2-ACYL-1-AL KYL-SN-GLYCERO-3-PHOSPHORYLCHOLINE

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1. Introduction

Hydrolytic cleavage of one of the two fatty acid ester linkages in various 1,2-diacyl-glycerophosphatides by phospholipase A from different origins is a well established reaction [1–5]. Snake venom, containing phospholipase A, specifically cleaves the ester linkage at the 2-position of phosphoglycerides, giving rise to unsaturated fatty acids and 1-acylglycerophosphatides [1,2]. These investigations have been performed with a number of 1,2-diacyl-glycerophosphatides as substrates, whereas only a few studies have appeared on the hydrolysis of pure 2-acyl-1-alk-1′-enyl- and 2-acyl-1-alkyl-glycerophosphatides by phospholipases A [6–8]. Experimental evidence has been presented that phospholipase C from Clostridium perfringens catalyzes the hydrolysis of choline-plasmalogen [9,10] and of 1-alkyl-2-acyl-sn-glycero-3-phosphorylcholine [11]. Conflicting results were obtained on the relative rates of hydrolysis of alkenylacyl-sn-glycero-3-phosphorylcholine and the corresponding 1,2-diacyl-derivative by phospholipase D of cabbage leaves. Lands and Hart [12] found that alkenylacyl-sn-glycero-3-phosphorylcholine was essentially inert as a substrate for cabbage phospholipase D, while Waku and Nakazawa [8] observed a significant activity of the enzyme towards choline-plasmalogen.

Abbreviations: acylalkenyl, 2-acyl-1-alk-1′-enyl; acylalkyl, 2-acyl-1-alkyl.

Investigating the effect of phosholipase A_2 from crotalus atrox venom on phosphatidylcholine, phosphatidylethanolamine and the corresponding plasmalogens, we found a higher affinity of the enzyme for pure phosphatidylcholine than for pure phosphatidylethanolamine [7]. Compared to the 1,2-diacyl-derivatives, the corresponding plasmalogens were cleaved more slowly by the phospholipase A [7]. Since methods are available now to prepare 1,2-diacyl-, alkylacyl- and alkenylacyl-sn-glycero-3-phosphorylcholine, specifically labelled with different unsaturated fatty acids at the 2-position, we extended our previous work on phospholipases A_1 and A_2 [5,7,13–15] on the action of phospholipase A_2 purified from crotalus atrox venom towards these specifically labelled compounds. In brief, it has been found that acylalkenyl- and acylalkyl-phosphoglycerides were hydrolyzed at almost similar rates by the enzyme. Compared to phosphatidylcholine, both acylalkenyl- and acylalkyl-sn-glycero-3-phosphorylcholine were cleaved to an extent of approximately 20 per cent only. Furthermore the hydrolysis rate depended upon the fatty acid moiety at the 2-position of the phosphoglycerides.

2. Experimental procedures

The labelled 1,2-diacyl-, 2-acyl-1-alk-1′-enyl- and 2-acyl-1-alkyl-sn-glycero-3-phosphorylcholines listed in the table 1 were prepared as described in detail elsewhere by Woelk and Porcellati [5] and Woelk et

Table 1
Specifically labelled glycerophosphatide preparations

Glycerophosphatide	Spec. activity (nCi/μmol)
1-acyl-2-[^{14}C]linoleoyl-sn-glycero-3-phosphorylcholine	15.6
1-alk-1′-enyl-2-[^{14}C]linoleoyl-sn-glycero-3-phosphorylcholine	9.4
1-alk-1′-enyl-2-[^{14}C]linolenoyl-sn-glycero-3-phosphorylcholine	10.8
1-alk-1′-enyl-2-[^{14}C]arachidonoyl-sn-glycero-3-phosphorylcholine	8.9
1-alkyl-2-[^{14}C]linoleoyl-sn-glycero-3-phosphorylcholine	6.2
1-alkyl-2-[^{14}C]linolenoyl-sn-glycero-3-phosphorylcholine	11.2
1-alkyl-2-[^{14}C]arachidonoyl-sn-glycero-3-phosphorylcholine	6.7

al. [14]. Hydrolysis of the phosphoglycerides, differing in the radical at the 1-position and labelled at the 2-position with different unsaturated fatty acids, by phospholipase A_2 from naja naja venom showed that the radioactivity was almost exclusively (96–98%) recovered in the fatty acids freed from the substrates, indicating that specific incorporation of the ^{14}C-labelled acids into the 2-position of the phosphoglycerides had occurred.

Phospholipase A_2 was purified from lyophilized crotalus atrox venom according to the method described by Wu and Tinker [16]. The incubation of 1 μmol of the different glycerophosphatides with the purified phospholipase A_2 was carried out in the ethereal system as described previously by Woelk and Debuch [7]. Chromatographic separation and isolation of the reaction products as well as radioactivity measurements were as indicated by Woelk and Porcellati [5]. Control tubes without enzyme were included in each experiment; each figure in the table represents the average of at least five experiments; their values varied by less than 10 per cent.

1,2-diacyl-, 2-acyl-1-alk-1′-enyl-, 2-acyl-1-alkyl-sn-glycero-3-phosphorylcholine, the corresponding lyso-compounds, and the fatty acids were separated by one-dimensional thin-layer chromatography on Kieselgel G (Merck, Darmstadt, Germany) as described elsewhere [13], using chloroform/methanol/water 65:25:4 (v/v/v) [17] as the solvent system. The purity of the substrates was examined by two-dimensional thin-layer chromatography as indicated elsewhere [5].

Phospholipid P was determined by a modified [18] procedure of Bartlett [19], plasmal (as dimethylacetal) according to Feulgen et al. [20], as modified by Klenk and Debuch [21]. Protein was determined according to Lowry et al. [22] with crystalline bovine serum albumin as a standard.

3. Results and discussion

1,2-diacyl-, 2-acyl-1-alk-1′-enyl- and 2-acyl-1-alkyl-sn-glycero-3-phosphorylcholine, specifically labelled at the 2-position with different unsaturated fatty acids, were prepared enzymatically using the acyltransferase system of rabbit sarcoplasmic reticulum (table 1). The substrate specificities of phospholipase A_2 purified from *Crotalus atrox* venom were compared by means of these compounds. As can be observed from table 2 acylalkenyl- and acyl-alkyl-phosphoglycerides were hydrolyzed at almost similar rates by the enzyme. Compared to phosphatidylcholine, both acylalkenyl- and acylalkyl-sn-glycero-3-phosphorylcholine were cleaved to an extent of approximately 20 per cent only (table 2). When 1-alk-1′-enyl-derivatives, esterified with different unsaturated fatty acids at the 2-position, were used as the substrates for phospholipase A_2, the enzyme preferentially liberated linolenic, linoleic and arachidonic acid in that order from the glycerophosphatides and thus behaved like phospholipase A_2 obtained from cobra venom (Woelk and Peiler-Ichikawa [23]). Table 2 shows furthermore that phospholipase A_2 purified from crotalus atrox venom preferentially removes those fatty acids from the 1-alkyl-2-acyl-derivatives that have the fewest double bonds, being decreasingly active with linoleic, linolenic and arachidonic acid linked to the 2-position of the acylalkyl-compounds.

Several investigations have appeared on the biosyn-

Table 2
Hydrolysis of various glycerophosphatides, differing in the radical at the 1-position and in the fatty acid constituent at the 2-position, by purified phospholipase A_2 from crotalus atrox venom

Substrate	Specific activity[a] (units/mg of protein)
1-acyl-2-[^{14}C]linoleoyl-sn-glycero-3-phosphorylcholine	151.4 ± 10.2
1-alk-1′-enyl-2-[^{14}C]linoleoyl-sn-glycero-3-phosphorylcholine	25.6 ± 1.8
1-alk-1′-enyl-2-[^{14}C]linolenoyl-sn-glycero-3-phosphorylcholine	34.8 ± 2.0
1-alk-1′-enyl-2-[^{14}C]arachidonoyl-sn-glycero-3-phosphorylcholine	19.8 ± 1.5
1-alkyl-2-[^{14}C]linoleoyl-sn-glycero-3-phosphorylcholine	32.4 ± 1.1
1-alkyl-2-[^{14}C]linolenoyl-sn-glycero-3-phosphorylcholine	26.1 ± 2.0
1-alkyl-2-[^{14}C]arachidonoyl-sn-glycero-3-phosphorylcholine	18.2 ± 1.4

[a] 1 unit of enzyme activity is defined as the amount of enzyme which hydrolyzes 1 μmole of the substrate × min^{-1} under conditions where zero-order kinetics apply. Specific activity is expressed in enzyme units × mg^{-1} prot.

thesis of plasmalogens [24–26]. Debuch et al. [24] presented experimental evidence that 1-alkyl-sn-glycero-3-phosphorylethanolamine was a direct precursor of plasmalogens. The authors [24,25] concluded from their experiments that the dehydrogenation of the ether bond to form the enol–ether bond occurred mainly at the stage of the lysophosphatides. However, only few information is available on the degradation of plasmalogens and the corresponding ether-derivatives. In this latter connection Warner and Lands [27] have shown that rat liver microsomes contain an enzyme catalyzing the hydrolysis of the vinyl ether linkage of choline–plasmalogen. Of the substrates tested, only the deacylated plasmalogen, 1-alkenyl-sn-glycero-3-phosphorylcholine, proved to be active, and the reaction products were free aldehyde and glycero-3-phosphorylcholine. These findings provide the experimental basis for understanding the metabolic breakdown of plasmalogens. Since the only active substrate contains a free hydroxyl group adjacent to the enol–ether bond, the metabolic breakdown of plasmalogen may first involve a phospholipase A_2 converting the plasmalogen into its corresponding lyso-derivative. Most recent studies by Woelk et al. [14] on the action of phospholipases A_2 obtained from human cerebral cortex and rat brain mitochondria, showed that both phospholipase A_2 hydrolyzed the 1,2-diacylphosphoglycerides more rapidly than the acylalkyl- and acylalkenyl-analogs. In contrast to what had been observed with the phospholipase A_2 purified from *Crotalus atrox* venom (table 2), the enzyme from rat brain mitochondria had a higher affinity for 1-alkyl-2-acyl- than for 1-alkenyl-2-acyl-sn-glycero-3-phosphorylcholine. These substrates, however, were hydrolyzed at similar rates by the human brain enzyme [14]. Experimental evidence had been presented by Woelk and Debuch [7] that 1-alk-1′-enyl-2-acyl-sn-glycero-3-phosphorylcholine or -ethanolamine in the enzyme assay of phospholipase A_2 from crotalus atrox resulted in competitive inhibition of the hydrolysis of the corresponding 1,2-diacyl-derivatives. Metabolic implications and probable significance of this plasmalogen dependent inhibition have been discussed elsewhere [5].

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